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Department of Biochemistry and Molecular Biology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA.
E-mail: Xin.Xiang@usuhs.edu

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Single-Molecule Imaging: A Collagenase Pauses before Embarking on a Killing Spree

Single-molecule tracking provides new insights into how an ATP-independent endo-proteolytic machine digests collagen fibrils during their remodeling.

Gwangrog Lee[1] and Taekjip Ha[2,*]

The extracellular matrix is a well-organized macromolecular platform that specifies the mechanical properties of connective tissues to maintain the cell shapes. Collagen is a major element of the extracellular matrix and is the most abundant protein in human tissues. Somewhat like the art of knitting, collagen is weaved into protein strings to form collagen fibrils, which then form a lattice (Figure 1A), which are highly resistant to proteolytic degradation. Over time, however, this highly stable scaffold must undergo remodeling during pathophysiological processes, such as wound healing, tumor progression, metastatic invasion, and host defense mechanisms [1]. Matrix metalloproteases (MMPs) are the endopeptidases in charge of degrading collagen fibrils, hence called 'collagenases', and their activities must be tightly regulated. Although it is now known that other types of processive proteases, e.g. ClpXP, use chemical energy derived from ATP hydrolysis to mechanically unfold protein structures before digestion [2,3], it has been a puzzle how MMPs can help remodel stable organizations of collagen without using additional energy sources.

Studying native collagen fibrils is difficult using traditional enzymology tools because the extended substrate is insoluble and heterogeneous. In 2004, a new approach of fluorescence correlation spectroscopy that examines molecular diffusion on a sub-micron scale was applied to the study of an MMP subtype, MMP1, and led to the proposal of a Brownian ratchet model; MMP1 diffuses on type 1 collagen but its Brownian motion is biased through a 'burnt bridge' effect caused by collagen proteolysis [4]. But, because of the difficulty in handing native collagen samples and the technical limitations of averaging over many molecules, the earlier study could not address how MMPs initiate and carry out the degradation of the native substrate. Now, in this issue of *Current Biology*, Sarkar *et al.* [5] report the use of single-molecule fluorescence imaging to shed new light on these issues and provide a major leap in our understanding of the multiple phases of native collagen degradation. Fluorescently labeled MMP1 proteins were added to native collagen fibrils immobilized on the sample cell surface and the motion of single MMP1 molecules on the fibrils was monitored in real time through total internal reflection fluorescence microscopy.

As anticipated, the authors found that MMP1 diffuses on the collagen fibrils. But direct imaging allowed them to show that the motion is one-dimensional (1D), occurring along the collagen fibril, but not across fibrils, raising the possibility that MMP1 uses 1D diffusional search to find the cleavage sites on the 3D collagen lattice. Interestingly, MMP1's 1D diffusion was not continuous but was punctuated by pauses. In fact, MMP1 spent ~90% of the time in paused states with only ~10% of time spent transiting between different pausing sites. As a result, these pauses dominate the overall diffusion timescales. One class of pauses followed a single exponential distribution of their lifetimes and occupied no special positions on the fibrils. The second class of pauses was longer in duration and had a distinct lag phase before escaping the paused state. Furthermore, statistical analysis showed that multiple sequential steps are necessary before the escape (Figure 1B). The molecular origin of these class II pauses that exhibit the lag phase is as yet unknown but these pauses are reminiscent of the activity of nucleic acid enzymes that can accumulate elastic energy through in multiple irreversible reactions before transitioning to a subsequent phase [6–8]. Furthermore, these class II pauses occur at periodic locations (see below).

As the enzyme escapes the class II pause site, it shows a so-called 'ballistic' behavior with a distinct bias in its initial motion along one fibril direction. This biased random walk was not observed with an active site mutant of MMP1, suggesting that the directional bias is related to the endopeptidase activity of MMP1. Furthermore, the ballistic behavior was observed at 37°C but not at 25°C. These observations led to an intriguing possibility that thermally induced local unfolding of collagen may allow MMP1 to initiate the collagenolysis. Upon initiation, cleavage reaction would bias the diffusion by burning the bridge behind so that subsequent diffusion appears ballistic along the collagen fibril. To obtain quantitative details of the collagenolysis the authors performed modeling and simulations. They found that only 5% of class II pauses result in the actual initiation of cleavage but this killing rampage is highly processive and, on average, 15 consecutive cleavage events result from one initiation event. MMP1 spends ~90% of its time at pausing states due to the inaccessibility of the cleavage sites, but once the first cleavage occurs, subsequent cleavages progress rapidly as the

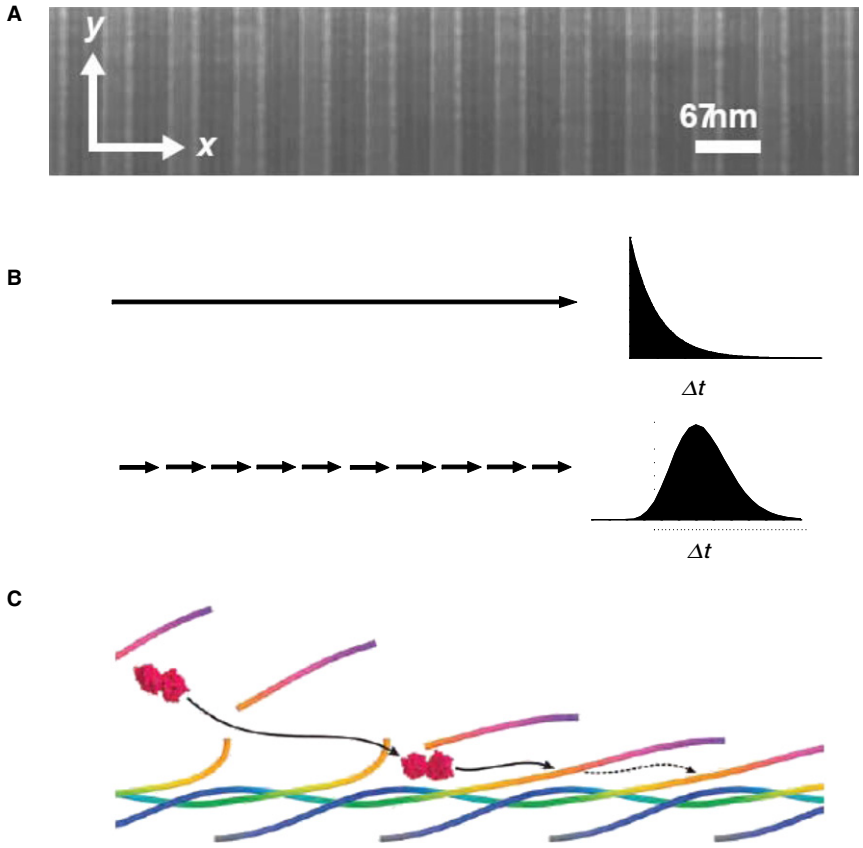

Figure 1. A collagenase pauses before launching onto a killing spree.

(A) Transmission electron microscopy image of a type I collagen fibril. (B) Two classes of pauses: class I pauses require a single rate-limiting step to exit and their dwell time histogram shows a single exponential decay (top); and class II pauses require a series of hidden steps to exit and as a result the histogram shows a lag phase (bottom). (C) Mechanism of processive cleavage by collagenase MMP1. The cleavage site is buried by the carboxyl terminus of the foregoing monomer. When MMP1 (purple) escapes from a pause site, it cleaves a collagen monomer, resulting in the subsequent exposure of the next cleavage site, allowing rapid and processive cleavage by MMP1. (Panels B and C from [5].)

next cleavage sites are exposed one at a time (Figure 1C).

If the exit from the paused states represents the initiation of collagen degradation, these pauses might therefore occur at defined positions on the collagen lattice. The authors found that the class II pauses indeed occur at periodic intervals of 1.3 and 1.5 μm and proposed that MMP1 preferentially recognizes specific 'hot-spots' that are periodically located. Because these periods do not match the 67 nm staggering in collagen organization or the 300 nm size of the collagen monomer, they may instead arise from the characteristic dynamic bending mode of the structural architecture of the fibril, governed by mechanical properties of collagen and its geometry. If so, the periodic intervals might also be used as a potential point of regulation because the dynamic bending mode might be more pronounced under mechanical tension to allow sensing by MMP1.

Overall, the data from Sarkar *et al.* [5] may answer the fundamental question of how MMP1 can digest the very stable collagen fibrils without using any external chemical energy sources. MMP1 may bind to the stable native collagen for a long time waiting for thermal fluctuations to initiate the collagenolysis. If the set-point of thermal activation is too low, then the enzymatic activity may lead to an unwanted and uncontrolled degradation with potentially disastrous consequences. How might be the collagenolysis regulated *in vivo*? Because protein binding to the substrate itself does not immediately lead to enzymatic activity, a very specific configuration or conformational change either on the protein or the substrate may be required for the formation of a catalytically active complex. The long pauses of class II might thus be used as a regulation point where the enzyme is bound but not activated yet so that its activity can be regulated, for example, by applying mechanical stress along the collagen fibrils. Indeed, mechanical stress influences the remodeling of extracellular matrix during embryonic development [9], aneurysm formation [10], atherosclerosis [11] and cancer metastasis [12].

Many enzymatic reactions comprise several phases such as initiation, elongation and termination as found for exonucleases [13], ribosomes [14] and RNA polymerases [15]. But multistep reactions are inherently difficult to dissect fully using bulk assays due to ensemble averaging. Using single-molecule fluorescent tracking combined with modeling and simulation, Sarkar *et al.* [5] have revealed detailed activities of a collagenase on native collagen fibrils. Even though the basis for the periodic hot spots where MMP1 binds and initiates the degradation reaction is presently unknown, this study paves a new way for future studies of other types of MMP–fibril interactions. Also, a similar approach may be used to study other matrix-degrading enzymes, such as cellulases, which are important for biofuels.

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[1]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea. [2]Howard Hughes Medical Institute & Department of Physics University of Illinois at Urbana-Champaign, 1110 West Green Street, Urbana, Illinois 61801, USA.
*E-mail: tjha@illinois.edu

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